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In re application of:

Masakazu Sato, et al.

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For: 20-HETE SYNTHASE INHIBITOR

DECLARATION UNDER 37 CFR §1.132

Commissioner for Patents
Alexandria, Virginia 22313-1450

Sir:

I, Noriyuki Miyata, hereby declare and state that:

1. I am a citizen of Japan, and have the following mailing address:

c/o Taisho Pharmaceutical Co. Ltd., 24-1, Takata 3-chome, Toshima-ku, Tokyo, Japan.

2. I was born in Kanagawa prefecture, Japan, on December 18, 1964. My present residential address is 2-1-6 Shinjuku-ku, Tokyo 161-0035, Japan.

3. I am one of the inventors of the subject application, and I am fully familiar with the subject matter thereof as well as the references relied upon by the Examiner in the prosecution of this application.

4. I received a bachelor's degree at the School of Pharmacy, Hoshi University, in 1987. In 1989, I received a master's degree in pharmacology from the Graduate School of Hoshi University. In 1994, I received a Doctorate in pharmacology from Hoshi

University. From 1995 to 1997, I was a postdoctoral fellow at the Department of Physiology, Medical College of Wisconsin (Prof. Allen W. Cowley).

5. I am an employee of Taisho Pharmaceutical Co., Ltd. From 1989 to 1997, I was engaged in research at the Department of Pharmacology, Research Center, Taisho Pharmaceutical Co., Ltd. (From 1995-1997, I was sent as a postdoctoral fellow to the Medical College of Wisconsin as described above). From 1997 to 2002, I was engaged in research as a research scientist at Medicinal Research Laboratories, Taisho Pharmaceutical Co., Ltd. Since 2002, I have been engaged in research as a senior research scientist and a group manager of Vascular Diseases and Diabetes Research Group, at Medicinal Pharmacological Laboratory, Taisho Pharmaceutical Co., Ltd.

6. I am a member of the following academic societies: American Physiological Society, High Blood Pressure Council (American Heart Association), Japanese Pharmacological Society, and Japanese Society for Circulation Research.

7. I have been involved in the following publications.

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8. I set forth hereinbelow the evidence that 20-hydroxyeicosatetraenoic acid (20-HETE) synthase inhibitor is useful for the treatment of cerebrovascular diseases.

I and co-researchers examined the effects of *N-hydroxyformamidine* derivatives, inhibitors of 20-HETE synthesis, on infarct volume in experimental ischemic stroke model, intracerebral hemorrhage model and on fall in cerebral blood flow after subarachnoid hemorrhage in rats to demonstrate the efficacy of these compounds in some cerebrovascular diseases, as explained below.

The data obtained from the following experiments led us to conclude that 20-HETE synthesizing enzyme inhibitor of *N-hydroxy-formamidine* will be a useful drug for cerebrovascular diseases including ischemic stroke, intracerebral hemorrhage and vasospasm after subarachnoid hemorrhage.

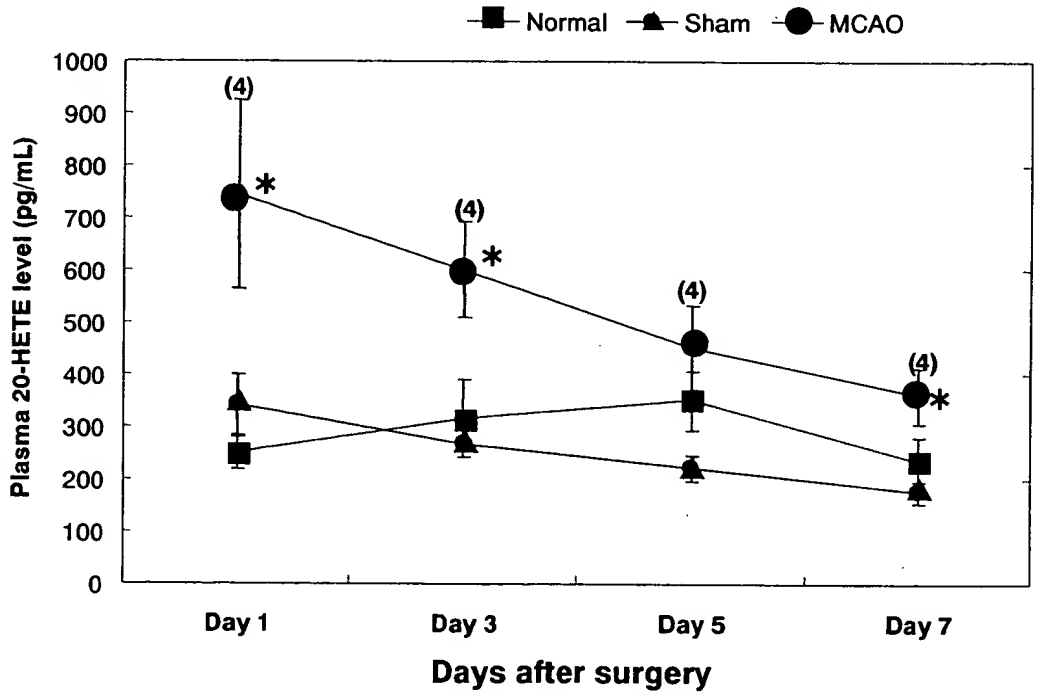
I. Arachidonic acid- 20-HETE pathway and physiological role of 20-HETE in cerebral circulation

Arachidonic acid can be metabolized by cytochrome P450 (CYP) to 20-HETE (Harder et al., 1995). In the rat kidney microsomes, there are three different CYP4A isozymes that can catalyze the omega-hydroxylation of arachidonic acid to 20-HETE. CYP4A1, CYP4A2 and CYP4A3 are all expressed in rat kidney (Ito et al. 1998). In the present Experimental Example, we used renal microsomes from spontaneously hypertensive rats to examine the effects of hydroxyformamidine derivatives on the 20-HETE synthesizing enzyme activities that were detected by the conversion of radio-labeled arachidonic acid to 20-HETE. The above-mentioned experiments is a golden standard method for measuring the 20-HETE synthesizing enzyme activity (Wang et al., 1998; Su et al., 1998, Miyata et al., 2001).
20-HETE is a potent vasoconstrictor eicosanoid which produced from arachidonic acid by CYP4A1, 4A2, 4A3 isoforms in rat (Gebremedhin et al., 2000) and cat cerebral artery (Gebremedhin et al., 1998). Vasoconstriction of cerebral artery causes reduction of cerebral blood flow. Decreases in cerebral blood flow were often observed in cerebral diseases such as subarachnoid hemorrhage (Kehl et al., 2002), stroke, traumatic brain

injury, dementia and alzheimer diseases. Reduced cerebroarterial vasoconstriction mediated through the inhibition of 20-HETE formation can improve the reduced cerebral blood flow observed in the above-mentioned cerebral diseases.
As a matter of fact, we demonstrated that 20-HETE levels in cerebrospinal fluid from rats after subarachnoid hemorrhage is 10 times higher than those from control rats. (Kehl et al., 2002). In addition, in rat and monkey stroke models, we found that plasma concentration of 20-HETE were significantly higher than that from control animals (Figure 1 and 2). In the above-mentioned stroke model, compound 302 reduced the infarct volume in rat stroke model (Figure 3) and improved the neurologic deficit in monkey stroke model (data not shown) with simultaneous reduction of plasma 20-HETE.

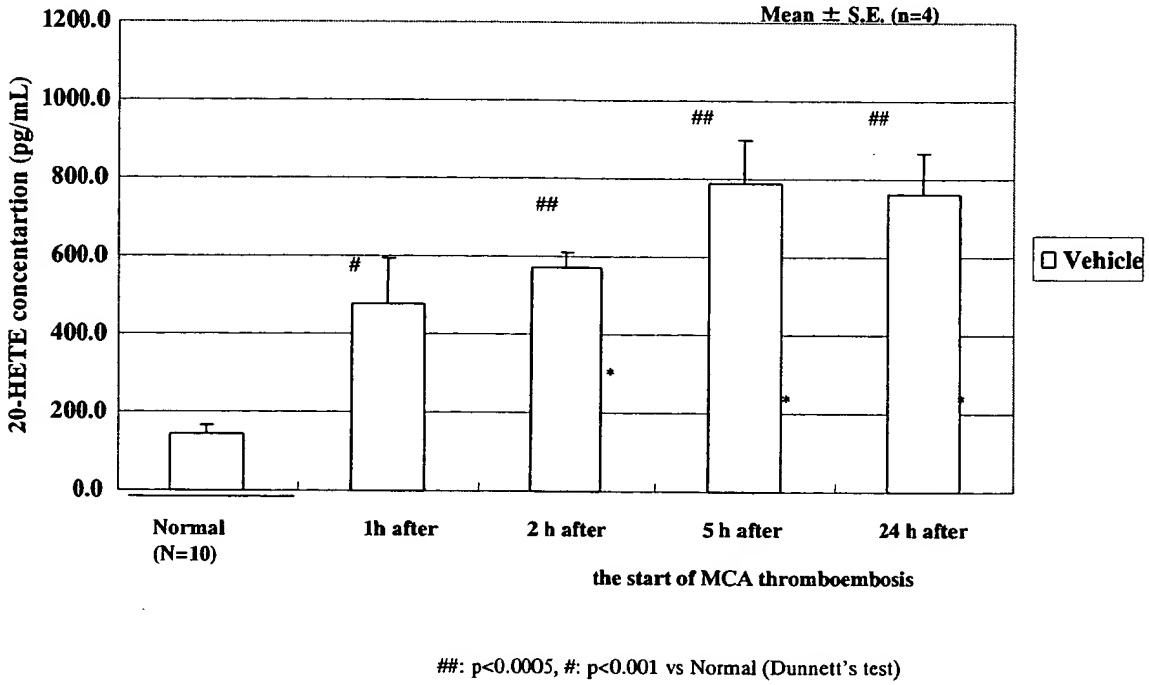
Therefore, increase in levels of 20-HETE might be involved in the pathological state of cerebrovascular diseases. From above-mentioned reports, an inhibitor of 20-HETE synthesis will be a good therapeutic drug for subarachnoid hemorrhage, stroke, traumatic brain injury, dementia and alzheimer diseases associated with decrease in cerebral blood flow.
We examined the effects of 20-HETE synthase inhibitor, N-hydroxyformamidine derivatives on the infarct volume of ischemic stroke, intracerebral hemorrhage, and regional cerebral blood flow after subarachnoid hemorrhage in rats to show the efficacy of the inhibitor of 20-HETE synthesis.

Figure 1: Plasma 20-HETE levels in rat transient middle cerebral artery occlusion model.



Transient MCAO was produced by the intraluminal suture method previously described by Nagasawa and Kogure (1989). Plasma levels of 20-HETE was measured by the LC/MS/MS techinique.

Figure 2: Plasma 20-HETE levels in monkey thromboembolic stroke models



##: p<0.0005, #: p<0.001 vs Normal (Dunnett's test)

Thromboembolic stroke was induced by the method of Kito et al. J Neurosci Methods 105 (2001) 45-53) . Plasma concentration of 20-HETE was measured by the LC/MS/MS technique.

II. Ischemic Stroke

Effect of compound 1, 302, 402 and 403 on the infarct volume in rat transient middle cerebral artery occlusion (MCAO) model

1. Methods

A catheter for sampling of blood and for measuring of blood pressure was surgically placed in the femoral artery one day before MCAO. Rats were anesthetized with 2 %(v/v) halothane and maintained with 1 %(v/v) halothane. The catheter (PE50, Becton Dickinson and Company) was placed in the femoral artery and was detained in hypodermically. The rectal temperature was maintained at 37.0±1.0 °C with a

heating pad (BWT-100, Bioresearch Center) during all procedures.

1.1 Middle Cerebral Artery Occlusion (MCAO)

Transient MCAO was produced by the intraluminal suture method previously described by Nagasawa and Kogure (1989). After induction of anesthesia with 2 % (v/v) halothane, the right common carotid artery, external cerebral artery, and internal cerebral artery were exposed through a midline neck incision and carefully separated from surrounding nerves and connective tissue. An 18-mm length of nylon suture (4-0) (Nitcho Kogyo Co., Ltd.) was coated with silicon (Xantopren VL plus, Hraeus Kulzer Dental Products Division) 5 mm length from its tip. This silicon-coated suture was introduced into the transected internal cerebral artery and advanced into the origin of the right middle cerebral artery. Successful occlusion was demonstrated by the appearance of neurologic deficits characterized by left hemiparesis. Rats that did not demonstrate left hemiparesis were excluded from analysis. At 60 minutes after the start of MCAO, rats were re-anesthetized with halothane and the sutures were withdrawn for reperfusion.

1.2 Drug Administration

Compound 302 (0.001, 0.01, 0.1, or 1.0 mg/kg), compound 1 (0.1mg/kg), compound 402 (0.1mg/kg) or compound 403 (0.1mg/kg) and vehicle (10 % hydroxy-propyl-β-cyclodextrin) were administered as a bolus intravenous injection at 60 minutes after the start of MCAO to groups of 9 to 22 rats (dose volume was 1.0 mL/kg). The study drugs were administered in a blind and randomized manner.

1.3 Calculation of Infarct Volume

The rats were killed by decapitation after anesthetization with diethyl ether. The brains were removed and cut into seven 2-mm thick coronal sections (from +4 mm to -8 mm bregma), using a rat brain matrix (RBM-2000C, ASI Instruments Inc.). The sections were immersed in a 2 % (w/v) TTC solution at 37 °C for 30 minutes, and subsequently fixed with 10 %(v/v) buffered formalin solution. Cortical and sub-cortical infarct areas were measured by NIH image version 1.62 (US-NIH). Cortical or sub-cortical infarct volume was calculated as follows:

$$\text{Infarct volume } (mm^3) = a + g + 2 \times (b + c + d + e + f)$$

a: the infarct area of the slice which is +4 mm from bregma
b: the infarct area of the slice which is +2 mm from bregma
c: the infarct area of the slice which is 0 mm from bregma
d: the infarct area of the slice which is -2 mm from bregma
e: the infarct area of the slice which is -4 mm from bregma
f: the infarct area of the slice which is -6 mm from bregma
g: the infarct area of the slice which is -8 mm from bregma

Total infarct volume was calculated as the sum of cortical and sub-cortical infarct volumes.

1.4 Statistical Analysis

Infarct volumes were calculated with Excel 97 (Microsoft Co.). The statistical evaluations were carried out with SAS (SAS Institute Inc.), version 6.12 or 8.2. Paired comparisons of infarct volumes was conducted with Dunnett's test. $p < 0.05$ was regarded as significant.

2. Results

2.1 Infarct Volume

Infarct volumes are graphically displayed in Figure 3. The total, cortical, and sub-cortical infarct volumes in the vehicle-treated group at 24 hours after the start of MCAO were 193.4 ± 18.6 mm^3, 136.3 ± 15.4 mm^3, and 57.1 ± 4.0 mm^3, respectively (n=22). The total infarct volumes in compound 302 0.001, 0.01, 0.1, and 1.0 mg/kg-treated groups were 149.7 ± 17.7 mm^3 (n=21), 125.5 ± 16.6 mm^3 (n=19), 124.7 ± 15.1 mm^3 (n=20), and 134.7 ± 17.6 mm^3 (n=21), respectively. The cortical infarct volumes in compound 302 0.001, 0.01, 0.1, and 1.0 mg/kg-treated groups were 99.0 ± 15.4 mm^3 (n=21), 80.4 ± 13.5 mm^3 (n=19), 79.2 ± 13.9 mm^3 (n=20), and 91.0 ± 13.5 mm^3 (n=21), respectively. The sub-cortical infarct volumes in compound 302 0.001, 0.01, 0.1, and 1.0 mg/kg-treated groups were 50.7 ± 3.5 mm^3 (n=21), 45.0 ± 3.5 mm^3 (n=19), 45.5 ± 2.6 mm^3 (n=20), and 43.7 ± 5.0 mm^3 (n=21), respectively (Figure 1).

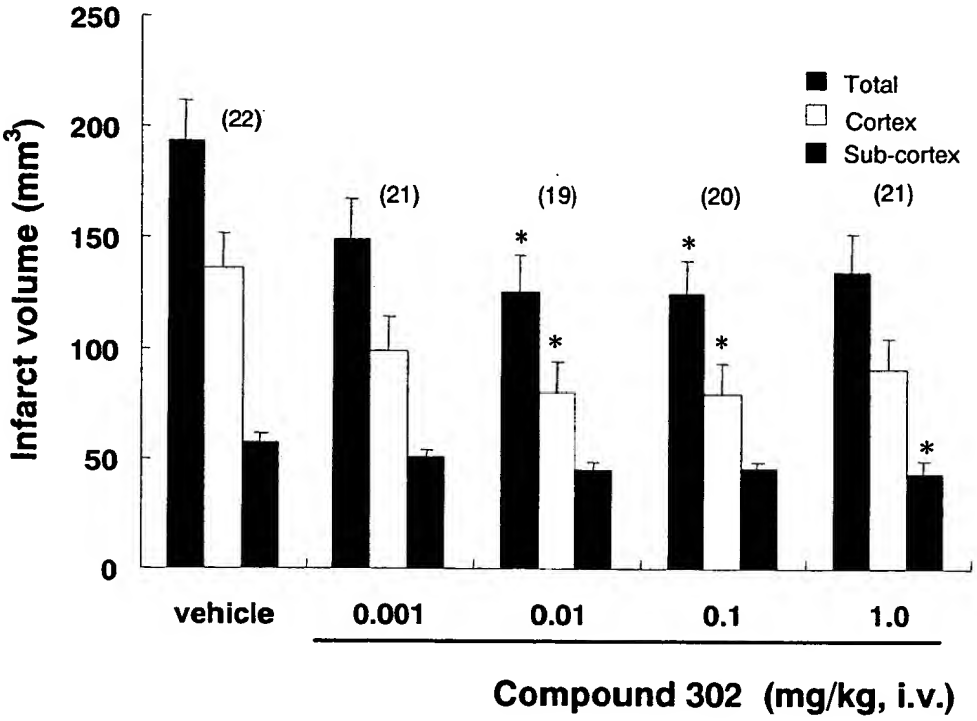
Total and cortical infarct volumes were significantly reduced compared with the vehicle-treated group ($p<0.05$) at 0.01 and 0.1 mg/kg compound 302, and sub-cortical infarct volume was significantly reduced at 1 mg/kg compound 302 compared to the vehicle control (Figure 3).

A bolus intravenous administration of Compound 1, 402 or 403 at a dose of 1.0mg/kg significantly reduced the total infarct volume in rat transient MCAO model as well as compound 302 (Figure 4).

3. Conclusion

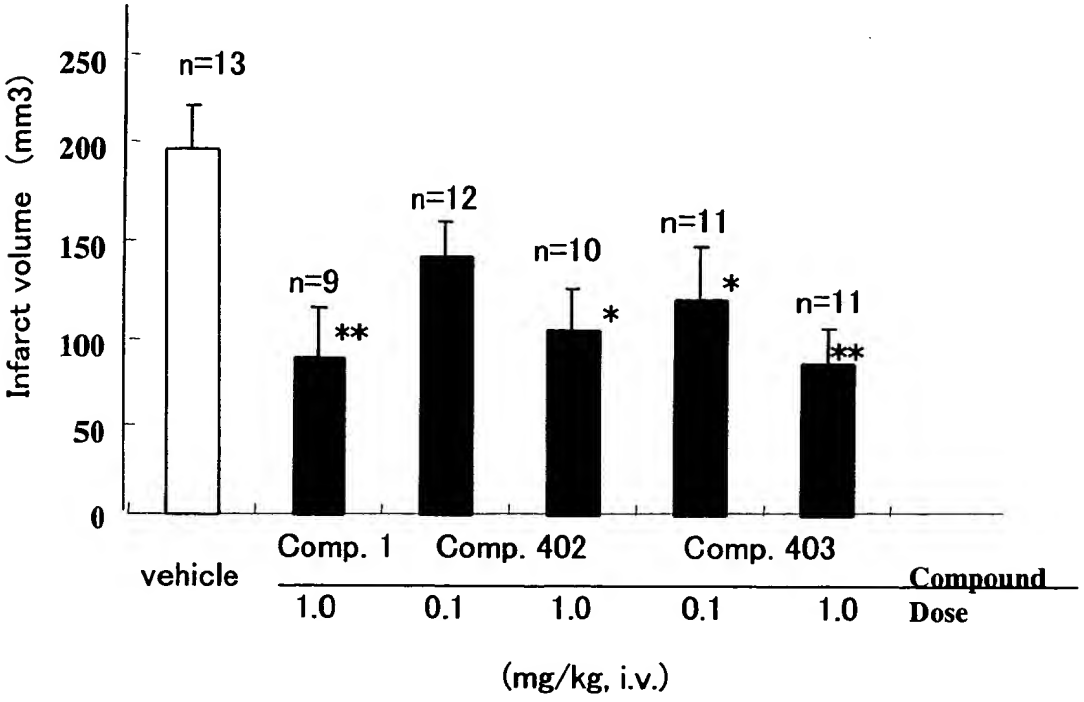
These results indicate that N-hydroxyformamidine derivatives have a beneficial effect in ischemic stroke.

Figure 3: The Effect of a Bolus Intravenous Injection of Compound 302 on Infarct Volume in the Rat Transient MCAO Model (Mean ± S.E.M.)



Compound 302 or the vehicle was administered by bolus intravenous injection at 60 minutes after the start of MCAO. Rats were killed at 24 hours after the start of MCAO, and brains were promptly removed. Each brain was cut into seven 2-mm thick coronal sections using a rat brain matrix. The sections were incubated in a 2 %(w/v) TTC solution at 37°C for 30 minutes. Infarct volume was measured and calculated as an integration of these areas. *$p < 0.05$ compared with vehicle-treated group (Dunnett's test). The number of animals is shown in parentheses.

Figure 4. The Effect of a Bolus Intravenous Injection of Compound 1, 402 and 403 on Total Infarct Volume in the Rat Transient MCAO Model (Mean ± S.E.M.)



* p<0.05, ** p<0.01 vs vehicle (Dunnett's test)

I. Intracerebral Hemorrhage Model

Effect of compound 302 on collagenase-induced intracerebral hemorrhage in rat

4. Methods

4.1 Animals

Male IGS strain rats (SPF: Charles River Japan, Inc. Atsugi, Kanagawa, Japan) that were 7 weeks old at the time of purchase were quarantined and acclimated for 6 days or more. During the acclimating period, the general conditions of animals were observed. Those confirmed as healthy and weighing 273 - 352 g on the day of test substance administration were used in the experiment. These animals were 8 weeks old at the time of administration. Throughout the experimental period, 2 to 3 animals were placed in each stainless steel cage. These cages were installed in a stainless steel hanger rack (Cary Ltd.) in a clean rearing room (Room No. 2 in VIII Area) whose environment was adjusted to the temperature of 23.4 - 26.1°C, relative humidity of 44.1 - 64.3%, ventilation frequency of 10 - 20 times/hour, and lighting duration of 12 hours (7:00 - 19:00).

The animals had free access to radioactively sterilized commercial solid food (F-2, Funabashi Farm Ltd., Funabashi, Chiba, Japan) and tap water (Kaizu-cho, Kaizu-gun, Gifu, Japan) through a drinking bottle. The food was analyzed by the manufacturer and Japan Food Research Laboratories (Shibuya-ku, Tokyo, Japan) and the drinking water was analyzed by Japan Environment Technology Center Ltd. (Ichinomiya, Aichi, Japan). The results indicated that the contaminants contained in the food and water were below the stipulated levels that might influence the study.

4.2 Materials and Equipment

4.2.1 Test Compound

Compound 302: 0.001 mg/mL
Compound 302: 0.01 mg/mL
Compound 302: 0.1 mg/mL

All test compounds were synthesized and prepared by Taisho Pharmaceutical Co., Ltd. and were transported under ice-cold conditions. All solutions were kept in airtight containers, protected from light, and refrigerated until the time of use. Compound 302 solutions were used within 2 weeks after preparation.

4.2.2 Vehicle

10 % (w/v) hydroxypropyl-β cyclodextrin (Lot No.1248008).

Vehicle was stored in a refrigerator.

4.2.3 Reagents

The collagenase solution was prepared at the time of use by dissolving collagenase (Type IV, 295 units/mg, Lot No. 109H8614, Sigma-Aldrich Japan Ltd.) in saline solution (Lot No. 9A83N, Ohtsuka Pharmaceutical Co., Ltd.) containing 1.4 U of heparin sodium (181 units/mg, Lot No. 88H0056, Sigma-Aldrich Japan Ltd.) to the concentration of 0.14 U/0.7 μL. After preparation, the solution was kept under ice-cold conditions until the time of use.

4.3 Experimental Methods

Experiment 1 (Determination of The Area of Cerebral Infarction Lesion)

Experimental Group	Test Substance	Compound 302 Dose	Dosing Solution Volume	Collagenase	Number of Animals
Sham	Vehicle	------	1 mL/kg	-------	10
Control	Vehicle	-------	1 mL/kg	0.14 U/0.7 μL/head	10
Compound 302 low	Compound 302	0.001 mg/kg	1 mL/kg	0.14 U/0.7 μL/head	10
Compound 302 middle	Compound 302	0.01 mg/kg	1 mL/kg	0.14 U/0.7 μL/head	10
Compound 302 high	Compound 302	0.1 mg/kg	1 mL/kg	0.14 U/0.7μL/head	10

4.4. Administration of compound 302

Compound 302 was administered as a bolus intravenous (IV) injection into the caudal vein using a 1 mL disposable syringe and a 27G injection needle. The dosing solution volume of compound 302 was calculated on the basis of body weight measured on the day of administration.

4.5 Preparation of Intracerebral Hemorrhage Model

The collagen-induced cerebral infarction model was prepared by the method reported by Del Bigio et al (1996). Briefly, the rat was anesthetized by intraperitoneal administration of 30 mg/mL Ravonal® (thiopental sodium for injection, Tanabe Seiyaku Co., Ltd.) at 0.4 mL/head, and then was immobilized in a brain stereotaxic device (SN-1 and SR-1, Narishige Mfg.). The scalp was incised to expose the cranial bone and a hole measuring 1 mm in diameter was bored through the skull using a drill. A 30-gauge needle was introduced through the burr hole into the caudate nucleus (3 mm lateral to midline, 0.2 mm posterior to bregma, depth 6 mm below the surface of the skull).

Using a syringe pump (Model 11, Harvard Apparatus, Inc.), 0.7 μL of the collagenase solution was injected in 5 minutes from a 10μL microsyringe. During the 5-minute injection period, a thermostat pad (TR-100, Neuroscience, Inc.) was placed under the rat to maintain the rectal temperature at 37.6°C. After injection of collagenase, the needle was withdrawn, the hole in the cranial bone was closed with bone wax (LUKENS®), the incision was sutured, and the head removed from the stereotaxic device. Except for the collagenase injection, the same procedure was performed in the sham animals.

4.6 Observation of Neurological Deficits

All surviving rats were assessed according to the report by Peeling et al. (1998), and the circling behavior, beam walking, and forelimb extension tests given at 24 hours after collagenase administration were taken as an index of motor dysfunction. Maximal dysfunction in each test was given a total score of 10. The observer was blind to the group assignment of the animals. The assessment criteria for circling behavior, beam walking test, and forelimb extension are shown below.

(1) Assessment of circling behavior

Ipsilateral voluntary circling behavior of rat (that is, toward the collagenase administration side, i.e. left side) was observed.

0: No circling behavior
1: Despite no voluntary circling behavior, shifted to the left.
2: Rotated to the left when urged to walk.
3: Occasional voluntary circling behavior.
4: Continuous voluntary circling behavior.

(2) Beam walking test

The rat was placed on a wooden beam (width 2.4 cm, length 70 cm, height 20 cm) and was made to walk.

0: Walked over the beam to the end.
1: Lost balance during the walk and almost fell.
2: Fell off the beam while walking over it.
3: Though unable to walk at all, remained on the beam for 1 minute or more.
4: Unable to walk at all and fell off the beam immediately.

(3) Evaluation of forelimb extension

The rat was hung by the tail and the condition of bilateral forelimbs was observed.

0: Complete stretching of bilateral fore limbs.
1: No bilateral or unilateral fore limb stretching.
2: Complete internal bending of bilateral fore limbs.

4.7 Determination of Infarct Volume

Rats from Experiment 1 were decapitated immediately after blood collection for blood

gas determination (i.e., at 24 hours after collagenase injection). The whole brain was extracted, and three sections measuring 2 mm in width were taken, using a brain slicer, with the site of the collagenase injection needle insertion as the center. These sections were incubated in a 2% (W/V) 2,3,5-triphenyltetrazolium chloride (TTC) solution for 30 minutes at 37°C using a shaker. After staining, the cerebral sections were stored in 10% buffered formalin.

The stained cerebral sections were coded as A, B, and C for each rat in ascending order. Each section was photographed with a digital camera (4900Z, Fuji Photo Film Co., Ltd.), and cerebral infarct area determined using NIH Image (Ver. 1.62).

4.8 Statistical Analysis

Each measured value was reported as mean ± standard error (S.E.M.). Using the sham group as the control group, the mean of each value was compared between the 5 groups. A significant difference in the value was assumed as a difference from the sham group. As the next step, the sham group was excluded and the remaining 4 groups were compared using the vehicle group as the control group.

The area of cerebral infarction lesion was analyzed with the nonparametric Dunnett's multiple comparison test. The motor dysfunction scores were assayed by Steel's test. Differences with a probability of 5% were considered to be statistically significant ($p<0.05$). For data analysis, Office 97 and Office 2001 (Microsoft Corporation), SAS ver. 6.12 (SAS Institute Japan Inc.) and EXSAS ver. 5.00 (Scientist) were used.

5. RESULTS

5.1 Infarct Volume

Mean infarct volume and cerebral infarction incidence are presented in Table 1.

Table 1: Effect of a Bolus IV Injection of compound 302 (Comp. 302) on Cerebral Infarct Area 24 Hours after Intrastriatal Injection of Collagenase in Rats

Sample (mg/kg)	Collagenase (μL/5 min/head)	Incidence of cerebral necrosis (%)		Necrotic area (mm^2)			
				A	B	C	Total
Sham	-	0 (0/10)	Mean	0.00	0.00	0.00	0.00
			S.E.M	0.00	0.00	0.00	0.00
Control	0.7	100 (10/10)	Mean	4.24	20.82 **	17.42 **	42.48
			S.E.M	1.58	2.13	2.07	5.14
Comp.302 0.001	0.7	100 (10/10)	Mean	6.53 **	18.76 **	12.82 **	38.10
			S.E.M	2.00	1.71	2.12	3.68
Comp. 302 0.01	0.7	100 (10/10)	Mean	3.35	15.94 **	10.82 **/#	30.10
			S.E.M	1.78	2.04	1.41	3.94
Comp. 302 0.1	0.7	100 (10/10)	Mean	2.42	13.13 **/#	11.75 **	27.30
			S.E.M	0.86	1.96	1.76	3.83

**P<0.01, significantly different from the Sham group (Dunnett's test).
#P<0.05, significantly different from the Control group (Dunnett's test).

The incidence of cerebral infarction in the vehicle control group as well as 0.001, 0.01, and 0.1 mg/kg groups of compound 302 was all 100%. The mean total areas of cerebral infarction lesion in the vehicle control group, and the 0.001, 0.01, and 0.1 mg/kg groups of compound 302 were 42.48, 38.10, 30.10, and 27.30 mm^2, respectively, indicating a dose-dependent decrease in the infarct area. Compared with the vehicle control group, a significant decrease ($p < 0.05$) in the total area of infarction was observed in the 0.1 mg/kg group.

5.2 Neurological Deficit

Tables 2 show the effect of compound 302 administered at 24 hours after collagenase injection on the neurological deficit score in Experiments 1 and 2.

At 24 hours after collagenase injection, the total scores in the sham group and control group were 0 and 7 respectively, and the total scores in the 0.001, 0.01 and 0.1 mg/kg groups of compound 302 were 7, 6, and 5, respectively. Neurological deficit scores were improved significantly by treatment with compound A at the dose of 0.1 mg/kg, compared with the vehicle control group.

Table 2: Effect of a Bolus Intravenous Injection of Compound 302 (Comp. 302) on Neurological Deficit Score at 24 Hours after Intrastriatal Injection of Collagenase in Rats

Sample (mg/kg)	Collagenase (µL/5 min/head)	n		Circling behavior	Beam walk	Forelimb extension	Total
Sham	-	20	Mean	0	0	0	0
			S.E.M	0	0	0	0
Control	0.7	20	Mean	2 **	4 **	1 **	7 **
			S.E.M	0	0	0	0
Comp. 302 0.001	0.7	20	Mean	2 **	3 **	1 **	7 **
			S.E.M	0	0	0	0
Comp. 302 0.01	0.7	20	Mean	2 **	3 **	1 **	6 **
			S.E.M	0	0	0	0
Comp. 302 0.1	0.7	20	Mean	1 **	3 ** ##	1 ** ##	5 ** ##
			S.E.M	0 #	0	0	0

**P<0.01, significantly different from the Sham group (Steel test).

##P<0.01, #P<0.05, significantly different from the Control group (Steel test).

6. CONCLUSION

Compound 302 (0.1 mg/kg, i.v.) significantly reduced infarct size and decreased neurological deficits induced by exposure of the brain to collagenase. These results indicate that compound 302 may have a beneficial effect in intracerebral hemorrhage.

III. Subarachnoid Hemorrhage Model

Effects of compound 302 on fall in cerebral blood flow after subarachnoid hemorrhage (SAH) in rats

7. Methods

7.1. Effect of compound 302 on fall in cerebral blood flow after SAH.

Experiments were performed on male Sprague-Dawley rats (9-12 weeks old). The rats were anesthetized with ketamine (Ketaject; 20mg/kg i.m.) and thiobutabarbital (Inactin; 50 mg/kg i.p.). A cannula was placed in the trachea, and the rats were artificially ventilated with a small-animal ventilator (SAR-830; CWE. Ardmore, PA, U.S.A.) with a mixture of 30 % O_2 in N_2. Cannulas were placed in the femoral vein for infusion of drugs and in the femoral artery for collection of arterial blood samples and measurement of mean arterial pressure (MAP). End-tidal P_{CO2} was maintained at 35 mmHg by adjusting the minute ventilation according to the reading of a CO_2 analyzer (Capstar-100 IITC, Woodland Hills, CA). Blood samples were collected at the beginning and end of the experiment and analyzed with a blood gas analyzer (ABL300; radiometer, Copenhagen, Denmark) to validate the end-tidal P_{CO2} readings. Anesthesia was maintained by administering additional amounts if thiobutabarbital (8 mg/kg, i.v.) as needed. Body temperature was maintained at 37 $\pm$ 1 °C with a thermoregulated heated pad, and the rats received an intravenous infusion of 0.9 % NaCl solution containing 1 % bovine serum albumin at a rate of 3 ml/hour to replace fluid losses.

Induction of SAH and measurement of cerebral blood flow. SAH was induced by injection of 0.3 ml of unheparinized autologous freshly drawn arterial blood into the cisterna magna with a modification of a posterior craniocervical approach as described previously by Delgado et al. (1985) and Solomon et al. (1985). The head of the rat was placed in a stereotactic head holder, and the atlantooccipital membrane was exposed by separating the overlying neck muscles in the midline. With the use of a stereomicroscope, a 30-gauge needle attached to a PE-10 catheter was inserted into the cisterna magna by penetrating the atlantooccipital membrane. A small hole was drilled in the bone overlying the left parietal cortex, and a PE-10 catheter pulled to a tip diameter of 100μm was inserted under the dura and cemented in place with cyanoacrylate fir monitoring ICP. A 3 x 5 mm area of the bone overlying the right parietal cortex was thinned using a hand-held drill until only a thin, translucent layer of cranial bone remained (thinned cranial window). A PF 102 laser-Doppler flow plobe was positioned over the cranial window, and regional cerebral blood flow (rCBF) was monitored with a PF-3 laser-Doppler flowmetry (Perimed, Stockholm, Sweden). After control measurements of rCBF were obtained, freshly drawn unheparinized arterial blood was infused into the cisterna magna at a rate of 30 μl/min over a 10-min period. This created a massive SAH that was confirmed in all rats at autopsy. Clotted blood was found overlying the cerebello-medullary junction posterior and the basal artery and the vessels of the circle of Willis on the ventral surface of the brain.

Pretreatment of rats with compound A. Experiments were performed in five separate

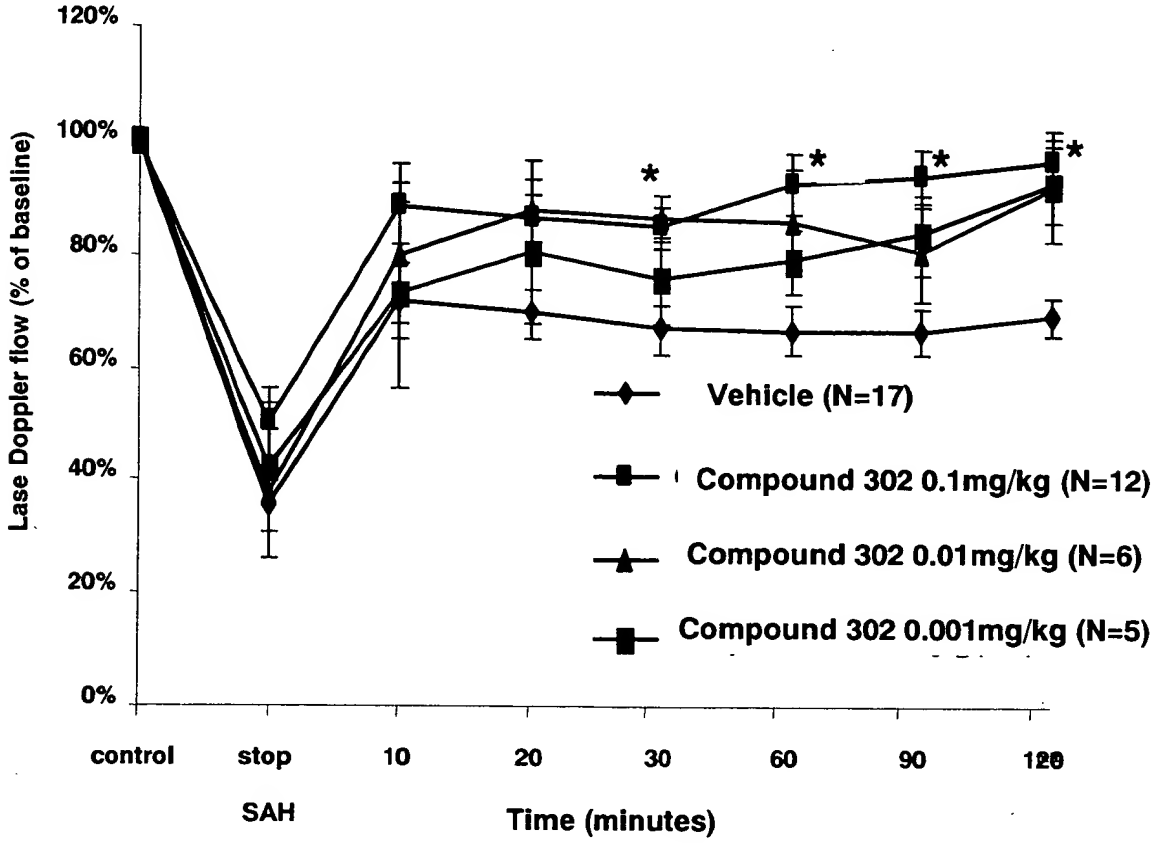
groups of rats. After surgery and a 30-min equilibration period, vehicle 11 % sulfobutylether-beta-cyclodextrin (SBE-Beta-CD; Group 1) or compound 302 (0.001, 0.01 and 0.1mg/kg, i.v.; Group 2) was given intravenously and rCBF was measured for 30 min. The average value of rCBF recorded over the last 5 min before induction of SAH served as the control value. SAH was induced, and rCBF was recorded over 2-min intervals at 10, 20, 30, 60, 90, 120 min after induction of SAH. The rCBF data are expressed as the percent change in flow from control. Another group of rats (Group 3) received an infusion of an equal volume of artificial cerebrospinal fluid (aCSF) instead of blood control for changes in cerebral blood flow that might be related to the infusion and accompanying increase in ICP alone.

Measurement of 20-HETE levels in CSF. At the end of many experiments, CSF (~100µl) was collected from the cisterna magna with a 1-ml syringe and a 30-gauge needle. CSF was also collected from additional sham-operated rats that did not receive an injection of blood into the cisterna magna. 20-HETE concentration in CSF was measured with a fluorescent HPLC assay as described previously (Maier et al., 2000). Briefly, 50 ng of an internal standard 20-hydroxyeicosa-6(Z), 15(Z)-dienoic acid (WIT0002) was added to the CSF samples (50 µl). The samples were extracted with 1 ml of ethylacetate and dried under argon. The lipid fraction was labeled with 20 µl of acetonitrile containing 34.4 mM 2-(2,3-naphthalimino)ethyltrifluoromethanesulfonate. *N,N*-diisopropylethylamine (10 µl) was added to catalyze the reaction. The sample was reacted for 30 min at room temperature. Excess dye was removed with a Sep-Pak Vac column (no. WAT054955; Waters, Milford, MA), and the samples were dried under argon, re-suspended in 100 µl of methanol, and analyzed on a reverse-phase HPLC column (Waters) with a fluorescence detector. The amount of 20-HETE in the sample was determined by comparing the area of the 20-HETE peak to that of the internal standard.

8. Results

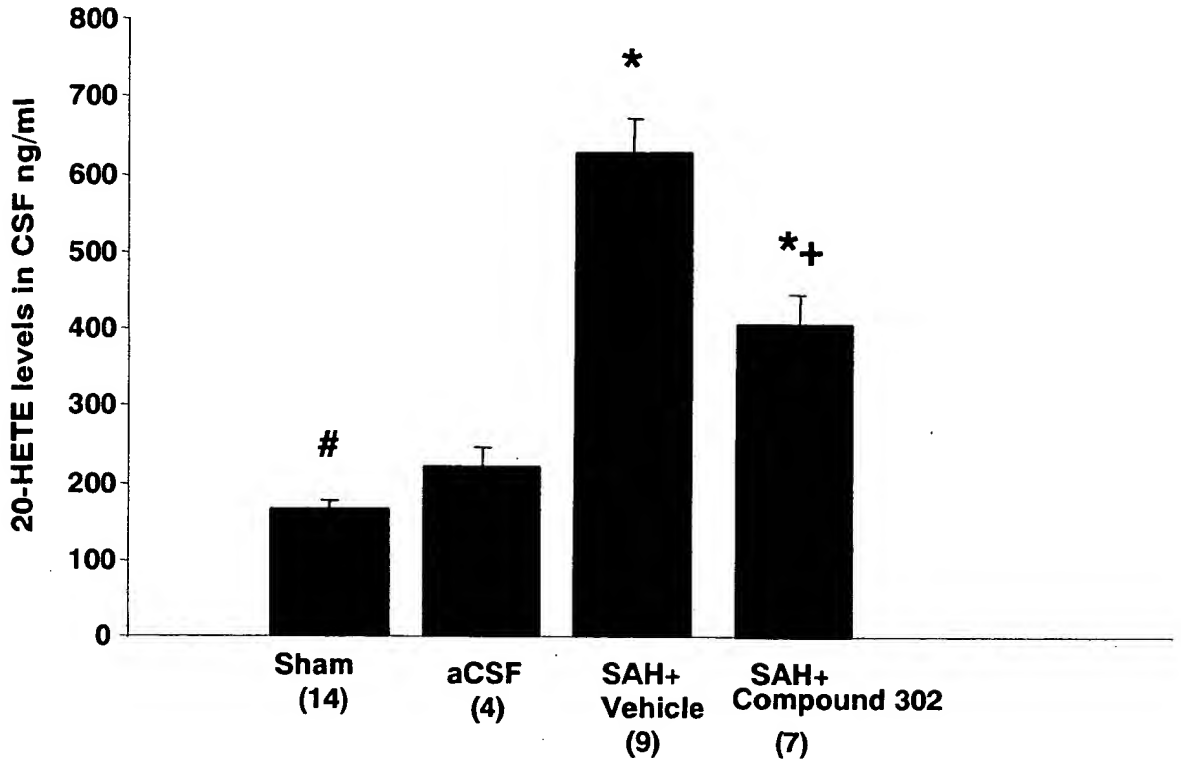
In vehicle-treated rats, cerebral blood flow measured with laser-Doppler flowmetry fell by 30 % 10 min after the injection of 0.3 ml arterial blood into the cisterna magna, and remained at this level for 2 hours. An intravenous bolus administration of compound 302 at doses of 0.001, 0.01 and 0.1 mg/kg reduced the fall in cerebral blood flow after SAH in a dose-dependent manner (Figure 5).

Figure 5: Effect of a Bolus Intravenous Injection of Compound 302 on fall in cerebral blood flow after subarachinoid hemorrhage in the rat (Mean ± S.E.M.)



These results indicate that compound 302 will be a useful drug for the treatment of fall in blood flow after subarachnoid hemorrhage.

Figure 6 Effect of compound 302 on the 20-HETE levels in cerebrospinal fluid from various groups of rats (Mean ± S.E.M.).



Rats that received artificial CSF into the cisterna magna did not change the 20-HETE levels in CSF (Figure 6). SAH model that received vehicle had a 3.8-fold increase in the concentration of 20-HETE in CSF 2 hours after SAH compared with the levels measured in sham-operated animals. Intravenous bolus administration of compound 302 markedly reduced the 20-HETE levels in CSF (Figure 6).

These results indicate that 20-HETE contributes to the fall in cerebral blood flow after SAH and compound 302, an inhibitor of 20-HETE synthase can improve the fall in cerebral blood flow after SAH.

Effects of compound 1 on fall in cerebral blood flow after subarachnoid hemorrhage (SAH) in rats

Recently, we have reported that an inhibitor of 20-HETE synthase, compound 1 (HET0016: *N-hydroxy-N'-(4-butyl-2methyl-phenyl)formamidine*) also improved fall in cerebral blood flow after subarachnoid hemorrhage in rats with concomitant decrease in 20-HETE levels in CSF (Please see attached paper: Kehl et al., Am. J. Physiol. 282, H1556-H1565, 2002).

9. Conclusion

These data clearly show that *N-hydroxyformamidine* derivatives of different structure were effective similarly for the fall in cerebral blood flow after SAH.

10. General Conclusion

In conclusion, we examined the effects of *N-hydroxyformamidine* derivative on the different types of cerebrovascular diseases such as ischemic stroke model, intracerebral hemorrhage model and subarachnoid hemorrhage model. The above-mentioned results clearly demonstrated that *N-hydroxyformamidine* will be a useful drug for the treatment of ischemic stroke, intracerebral hemorrhage and vasospasm after subarachnoid hemorrhage.

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9. I understand fully the content of this declaration.

10. I, Noriyuki Miyata, the undersigned declarant, declare further that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true, and further, that these statements are made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001, of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Respectfully submitted,

Noriyuki Miyata

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